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Molar Absorptivities of Bilirubin (NIST SRM 916a) and Its Neutral and Alkaline Azopigments

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Three laboratories in the U.S. and two in the Netherlands determined molar absorptivities (ϵ) of Standard Reference Material (SRM) 916a Bilirubin from the National Institute of Standards and Technology. In caffeine reagent the average ϵ values were 50 060 and 48 980 $L \cdot mol^{-1} \cdot cm^{-1}$ at 432 and 457 nm, respectively. The ϵ value of the blue azopigment, obtained with the Reference Method for total serum bilirubin, was 76 490 $L \cdot mol^{-1} \cdot cm^{-1}$ at 598 nm. When the addition of alkaline tartrate was omitted, the molar absorptivity of the red azopigment was 56 600 $L \cdot mol^{-1} \cdot cm^{-1}$ at 530 nm.

Additional Keyphrases: *Reference Materials · spectrophotometry · standardization*

Molar absorptivity values for bilirubin and its azopigments are utilized for evaluating the accuracy of bilirubin concentration in standard solutions, for assigning bilirubin values to calibrators and controls, and for assessing the purity of crystalline bilirubin preparations. Although molar absorptivity (ϵ) values for the azopigment of Standard Reference Material (SRM) 916 at 598 nm have been reported in the past (*1*), we felt it important to establish the molar absorptivity values for the current SRM 916a: this being the second SRM bilirubin produced by the National Institute of Standards and Technology (NIST, formerly the National Bureau of Standards), the possibility of its differing from SRM 916 could not be excluded.[7]

This collaborative study was conducted by the Working Group on Bilirubin of the American Association for Clinical Chemistry Committee on Standards. The purpose of the study, which was performed by all participants according to a detailed protocol, was to establish ϵ values for the alkaline and neutral azopigments at 598 nm and 530 nm, respectively, and for bilirubin in caffeine reagent at 432 and 457 nm, respectively.

Materials and Methods

Spectrophotometers. All instruments used had a bandpass of <2 nm. Checks for wavelength calibration, photometric accuracy and linearity, and photometric drift were performed as described previously (*2*); all instruments met the criteria.

Pipettes and cuvets. Class A volumetric pipettes and 10 (± 0.01)-mm-pathlength square silica or glass cuvets were used. Within each laboratory, the same cuvet was used for all measurements and was always oriented in the same direction.

Materials for preparing bilirubin solutions. Each laboratory was provided with one vial (100 mg) of SRM 916a and

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[7] Nonstandard abbreviations: SRM, Standard Reference Material; NIST, National Institute of Standards and Technology; and BSA V, bovine serum albumin, Cohn Fraction V.

20 g of bovine albumin Fraction V (BSA V, reagent grade; ICN Biochemicals, Inc., Costa Mesa, CA 92626).[8]

Bilirubin solutions. Bilirubin stock standard solutions (340 μmol/L; 20 mg/dL) were prepared as previously described (*1*). Standard blank solutions were prepared in the same way, except that bilirubin was omitted. These blank solutions were used to dilute the stock standard solutions and served as sample blanks in various bilirubin assays.

Three laboratories prepared stock solutions on two occasions, one laboratory on three, and another on five. The bilirubin solutions were analyzed on the day of preparation and as expeditiously as possible.

To compare the two bilirubin SRMs (916 and 916a), one laboratory prepared solutions of each SRM in BSA V (Armour Pharmaceutical Co., Biochemical Division, Kankakee, IL 60901) on the same day and analyzed them in a single run. The same experiment was performed on another occasion except that crystalline BSA (SRM 926) from NIST was used instead of BSA from Armour.

Methods. We used the Reference Method for total bilirubin in serum (*1*) to establish the molar absorptivity of the alkaline azopigment at 598 nm. The ε value of the neutral (red) azopigment at 530 nm was established by the same method, but the addition of alkaline tartrate was omitted. The absorptivity of bilirubin at 432 and 457 nm was determined by the method of Vink et al. (*3*).

Bilirubin stock solutions were analyzed in triplicate and standard blank solutions in duplicate. Twofold and fourfold dilutions of the stock standard were also analyzed in the same run. Bilirubin solutions were prepared and analyzed under subdued light.

Results

The raw data from this study on the molar absorptivity of bilirubin and its azopigments are listed in Table 1. Molar absorptivities were calculated from the absorbance values of the 340 μmol/L bilirubin standards. The 170 and 85 μmol/L solutions were analyzed only to ensure that there were no deviations from linearity because of improperly prepared reagents; data for the 170 and 85 μmol/L solutions are not shown. Because of possible dilution errors, the absorbance values of the diluted bilirubin solutions were not used in establishing molar absorptivities.

Each laboratory tended to agree with itself better than with the other laboratories, indicating the existence of both within- and between-laboratory components of imprecision. Separate statistical analyses were made for each wavelength, and four weighted molar absorptivities were obtained. Averages from each laboratory rather than original measurements were used because not all of the five laboratories reported the same number of measurements. The average ε value from each laboratory was weighted as the inverse of the variance of the average. The variance that was used for each average depends, in turn, on the within- and between-laboratory components of variance as well as the number of measurements (*4*). The weights determined from the data were all nearly equal because the between-laboratory component tended to be the largest source of variability. Expressed on a relative basis, the weights varied from a minimum of 18% to a maximum of 25%.

[8] Trade name disclaimer: Certain commercial equipment or materials are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

Table 1. Uncorrected Data for Molar Absorptivities (ε) of Bilirubin (SRM 916a) and of Its Neutral and Alkaline Azopigments

	ε, $L \cdot mol^{-1} \cdot cm^{-1}$			
	Caffeine reagent		Reference Method	
Lab.	432 nm[a]	457 nm[a]	530 nm[b]	598 nm[b]
1	48 783	48 094	—	75 456
	48 842	48 195	55 891	75 643
	48 730	48 090	55 530	74 470
	48 826	48 100	55 992	75 253
	48 671	48 013	55 573	74 913
2	49 440	47 040	53 830	74 180
	50 340	47 780	54 420	74 630
3	49 200	48 527	55 820	75 928
	48 739	47 952	55 428	75 546
4	49 634	48 904	56 754	75 128
	49 663	48 702	56 651	75 199
	49 761	49 058	—	75 609
5	48 610	47 917	55 746	74 672
	48 890	48 200	56 837	76 009
Weighted average	49 210	48 140	55 700	75 190
s_w	250	250	380	480
s_b	510	500	940	350

[a] Bilirubin. [b] Azopigments.

Also listed in Table 1 are the weighted averages and the within-laboratory (s_w) and between-laboratory (s_b) components of the standard deviation at each of the four wavelengths. Because the individual within-laboratory components of variance were quite similar, a better estimate was obtained by using an average (pooled) within-laboratory variance; s_w in Table 1 is the square root of the pooled variance. The between-laboratory standard deviation (s_b) was derived by an iterative calculation (*4*).

Because the purity of SRM 916a is 98.3% (*5*), the weighted averages were divided by 0.983 to obtain the corresponding corrected values (Table 2). Also shown in Table 2 are the standard deviations of the averages and the standard deviations of a single measurement ($\sqrt{s_w^2 + s_b^2}$). Interestingly, the Reference Method gave the smallest

Table 2. Corrected Molar Absorptivities and Standard Deviations of Bilirubin (SRM 916a) and of Its Neutral and Alkaline Azopigments

	ε, $L \cdot mol^{-1} \cdot cm^{-1}$			
	Caffeine reagent		Reference Method	
	432 nm[a]	457 nm[a]	530 nm[b]	598 nm[b]
Weighted average	50 060[c]	48 980	56 660	76 490
SD of average	240	240	440	210
SD of single measurement	580	570	1 030	610
CV of average, %	0.5	0.5	0.8	0.3
CV of single measurement, %	1.2	1.2	1.8	0.8

[a,b] Same as in Table 1.

[c] These averages have been divided by 0.983 to correct for the purity of bilirubin (98.3%) in SRM 916a.

imprecision (CV = 0.3%), but the same method without the addition of alkaline tartrate gave the largest imprecision (CV = 0.8%). However, all of the CVs are considered acceptably small.

Note: The standard deviation of the average ϵ value for SRM 916a at a particular wavelength estimates the variability of the averages one might obtain if the interlaboratory exercises were repeated numerous times within similar laboratories. The standard deviation of a single measurement estimates the variability of measurements one might obtain if a number of laboratories that are similar to those in the current study each made and submitted a single measurement result.

Molar absorptivities of SRM 916 and 916a obtained from bilirubin solutions prepared in BSA V on the same day and analyzed in a single run are shown in Table 3. Absorptivity values of SRM 916 and 916a prepared in SRM 926 (BSA from NIST) were obtained on a different day.

Discussion

The corrected average molar absorptivity of the SRM 916a azopigment at 598 nm, 76 500 $L \cdot mol^{-1} \cdot cm^{-1}$, listed on the SRM 916a certificate is almost identical (within 0.3%) to that reported for SRM 916; the reported SRM 916 ϵ value of 75 500 (*1*) becomes 76 260 when corrected for nonbilirubin impurities (*6*). The currently measured ϵ value for the SRM 916 (Table 3) is also in good agreement with that for the SRM 916a. The standard deviations for a single measurement of the ϵ values at 432, 457, and 598 nm are of the same magnitude (Table 2); the larger SD at 430 nm is due to the substantially lower ϵ value obtained in laboratory no. 2 (Table 1).

We thought it would be important to establish the molar absorptivity of the azopigment at 530 nm because in most clinical analyzers the Jendrassik–Gróf method (*7*) has been simplified by omitting the addition of alkaline tartrate, and the azopigment absorption maximum under those conditions is near 530 nm.

Establishing the molar absorptivity of bilirubin has been for many years an exercise in futility because in the commonly used buffers (e.g., borate, phosphate, Tris) its value is dependent on the protein matrix (*8, 9*) and, even within the same matrix, e.g., human serum or human serum albumin, the variation was unacceptably high (*1*). However, the recent observation by Vink et al. (*3*), that in the presence of caffeine reagent the spectrum and absorptivity of bilirubin are independent of the protein matrix, has made it possible to determine the ϵ value of unconjugated bilirubin with a high degree of precision. In a caffeine reagent, protein matrix has no effect on the spectrum of bilirubin because caffeine causes dissociation of bilirubin from its albumin binding sites (*10*). The two absorption maxima at 432 and 457 nm are most likely due to unbound bilirubin kept in solution as a complex with caffeine (*11*). Knowledge of the molar absorptivity of bilirubin at 432 and 457 nm is important for at least two reasons: it may serve as a second test (the first being the ϵ value of the azopigment at 598 nm) for assessing the accuracy of bilirubin standards and calibrators, and it may be used to measure bilirubin in sera from neonates, in whom conjugated hyperbilirubinemia is uncommon.

We believe this was a successful study because it established ϵ values of bilirubin and its azopigments within narrow limits. The overall CV of 1% (except 2% at 530 nm) for a single measurement—which includes errors in weighing, transferring the bilirubin into a flask, pipetting, and measuring absorbance in five different spectrophotometers—is remarkably small for an interlaboratory study, especially because there was no comparison standard (calibrator) available.

To ensure that bilirubin standard solutions have been prepared properly, we recommend that (*a*) the amounts of bilirubin weighed be corrected for the 98.3% purity of bilirubin in the SRM 916a, (*b*) standard solutions be analyzed by the Reference Method, and (*c*) the ϵ value of the azopigment be calculated at 598 nm. A bilirubin standard solution should be considered accurate if the ϵ value of its azopigment at 598 nm lies between 75 300 and 77 700 $L \cdot mol^{-1} \cdot cm^{-1}$.

We also recommend that, in addition to the molar absorptivity at 598 nm, ϵ values at 432, 457, and 530 nm be determined, because they can provide valuable information on the accuracy of bilirubin standard solutions.

Note: We followed the accepted convention for calculating the ϵ value of the azopigment by disregarding the fact that 1 mol of bilirubin yields 2 mol of azopigment.

Table 3. Comparison of Molar Absorptivities of SRM 916 with Those of SRM 916a[a]

	ϵ, $L \cdot mol^{-1} \cdot cm^{-1}$			
	Caffeine reagent		Reference Method	
	432 nm[b]	457 nm[b]	530 nm[c]	598 nm[c]
SRM 916 in BSA V	49 030	48 380	—	76 370
SRM 916a in BSA V[d]	49 620	48 920	—	76 780
SRM 916a in BSA V[d]	50 500	49 790	57 590	76 440
SRM 916 in SRM 926	49 310	48 690	56 790	76 210
SRM 916a in SRM 926	49 680	49 020	56 860	76 980
SRM 916, average	49 170	48 540	56 790	76 290
				(76 260)[e]
SRM 916a, average	49 930	49 240	57 220	76 730
	(50 060)[f]	(48 980)[f]	(56 660)[f]	(76 490)[f]

[a] Corrected for the nonbilirubin impurities of the SRMs, i.e., SRM 916, 1.0% (purity 99%); SRM 916a, 1.7% (purity 98.3%).
[b,c] Same as [a,b] in Table 1.
[d] Two separate preparations of SRM 916a in BSA.
[e] Result reported in reference 1.
[f] Averages from five laboratories, this study (Table 2).

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